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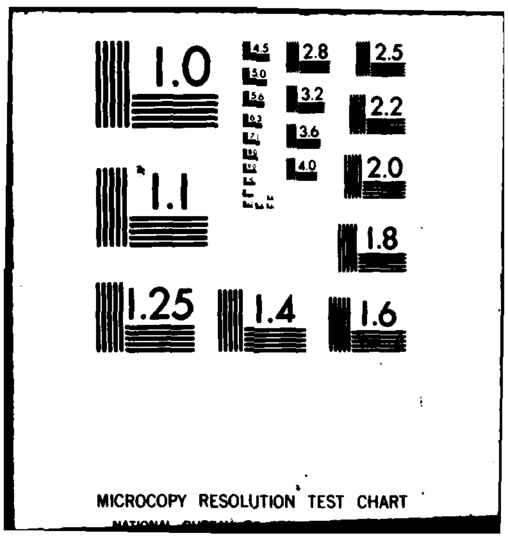
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TWO NOVEL METHODS FOR THE PREPARATION OF ERYTHROCYTE STROMA FREE FROM HAEMOGLOBIN COMPARED TO THE CONVENTIONAL CENTRIFUGATION TECHNIQUE.

H.D. Crone, M. Poretski and M.P. Bladen

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The removal of haemoglobin from haemolysed erythrocyte stroma by washing over a membrane filter or by passage through a column of porous gel was compared to the removal achieved by washing in the centrifuge. The column method was preferable for an initial rapid clean-up, which could be followed by a further pass through the column, or by centrifugation to concentrate the suspension. Membrane filtration was useful when total recovery of a small sample of stroma was required.

C O N T E N T S

TWO NOVEL METHODS FOR THE PREPARATION OF ERYTHROCYTE STROMA FREE FROM HAEMOGLOBIN COMPARED TO THE CONVENTIONAL CENTRIFUGATION TECHNIQUE

1. INTRODUCTION

The membrane of the mammalian erythrocyte is used widely as a model membrane for structural and functional investigations. The membranes are prepared by lysis of the erythrocytes in hypotonic media and then washed free of haemoglobin (Hb) by centrifugation at pH 7.4 in low ionic strength buffer solutions [1]. This method has been used in this laboratory for a number of years but since repeated washing in the centrifuge is a lengthy process, we have recently investigated other ways of removing the haemoglobin. We have found that the stroma is best prepared by rapid removal of the Hb immediately after haemolysis; prolonged contact of the membranes with the Hb solution encourages adsorption of the pigment. If the suspension is stored frozen with residual Hb present, a granular association of membranes and Hb is obtained on thawing.

This Note describes removal of the haemoglobin by a membrane filtration method and by a gel column method, and compares them with the centrifugation procedure.

2. METHODS

Freshly collected human blood was diluted into 150 mM NaCl, 10 mM Tris HCl, pH 7.4 and centrifuged to precipitate the cells. The latter were washed three times with the isotonic buffer, then haemolysed by suspension in an excess of 10 mM Tris HCl buffer at pH 7.4. The stroma was then washed free of haemoglobin by repeated centrifugation, or subjected to the two alternative procedures below. All operations were performed at 5°C.

The membrane filtration method consisted of stirring the haemolysate above a membrane of pore size sufficiently small to retain the membrane fragments but porous enough to allow rapid passage of the washing medium. Millipore type HA filters of 0.45 µm pore size were cut to fit into an Amicon model 52 diafiltration cell. The cell was loaded with 40-50 mL of

haemolysate, stirred and subjected to light pressure (< 30 KpA) by a peristaltic pump supplying air to the inlet. When the volume in the cell was reduced to 10 mL, 10 mM Tris buffer, pH 7.4, was pumped into the cell and washed through the filter at 20 mL. h^{-1}, until the effluent was nearly colourless. Further concentration of the retentate was performed when necessary by pumping in air rather than buffer solution. The stroma from 6 mL of whole blood could be washed over a 40 mm diameter filter with 120 mL of buffer solution in 400 min, to reduce the Hb content to < 0.5% of the initial value. No loss of stromal material, monitored as acetylcholinesterase (AChE) activity, was found. When desired, the washed suspension retained in the cell was solubilized by the addition of a suitable concentration of Triton X-100, and the effluent was then collected for further examination.

The column method of washing the stroma was based on the principle of passing the suspension through a bed of beaded, porous gel, so that the membrane particles passed rapidly between the beads and were eluted in the exclusion volume, but all the soluble material including the Hb entered the gel interior and was eluted later. A column of 7 cm^2 cross-sectional area was packed with cross-linked agarose of bead diameter 40-210 μm (Sepharose CL-6B, Pharmacia Fine Chemicals) to a volume of 37 mL, using 10 mM Tris HCl pH 7.4 as the buffer solution. A sample of 10 mL of the haemolysate (equivalent to the cells from 2.5 mL of whole blood) was applied to the column, and eluted with the buffer at a flow rate of 24 $mL.h^{-1}$. Fractions of 1.7 mL were collected and analysed for AChE [2], haemoglobin (absorbance at 412 nm) and turbidity (apparent absorbance at 700 nm). Figure 1 shows that the stroma (as represented by AChE activity and turbidity) was separated from the bulk of the Hb, and was eluted as a near square pulse not much wider than the initial 10 mL applied. From the results it can be shown that 92% of the AChE could be recovered with 11.5% of the Hb, or 60% AChE with 1% of Hb. Recovery was therefore dependent on the degree of separation desired. the stromal fraction could thus be washed in 70 min, and then concentrated by centrifugation or membrane filtration for further examination. A faster elution rate (50 $mL.h^{-1}$) through the column resulted in distortion of the gel bed as the sample passed through. It was desirable to repack the column frequently, as the pressure difference occurring as the membrane fraction passed through tended to deform the gel beads and pack them more tightly.

3. DISCUSSION

The centrifugation method for washing the membranes has the advantages of simplicity of operation and of being concentrative. Its disadvantages are that a relatively long time is necessary (for example 4h to complete 4 washes), it is not particularly efficient, it encourages coagulation of the stroma, and the capital cost is high. Membrane filtration is very efficient, concentrative and of low capital cost, but it is relatively slow. Problems can arise due to clogging of the membrane and coagulation can also occur. The column method is rapid, of low cost, and no tendency towards coagulation of the stroma exists. On the other hand the sample is recovered slightly diluted and requires concentration by another method. Efficient separation is achieved at the expense of yield. However, the incompletely

separated Hb may be removed by a second pass of the stromal suspension through the column, resulting in a high yield of a clean suspension.

When the above factors are considered, the membrane filtration method is preferable if full recovery of a small sample is necessary (e.g. if the membranes have been labelled in some way). For general isolation of moderate quantities, the gel column procedure followed by centrifugation to concentrate the suspension is the most rapid and effective method. Our experience is that the gel column produces the best suspension, in terms of freedom from the coagulated, sticky lumps often formed by the other methods. Thus centrifugation, membrane filtration and freezing - thawing all concentrate the membrane fragments and encourage association.

Both the novel methods described can be scaled up to handle larger quantities of stroma. The critical design feature of the membrane method is to achieve efficient stirring as close as possible to the filter surface. For the column, a uniform, evenly - packed bed of gel is necessary together with a column design allowing even loading on the top and uniform drainage from the bottom.

It is recommended that researchers in membrane science should consider adapting these methods to their particular needs, as alternatives to the universally used but not always efficient centrifugation.

4. CONCLUSION

Two methods are described for the removal of haemoglobin and other soluble compounds from erythrocyte membrane fragments, as alternatives to centrifugation. Continuous washing of the suspension held by a Millipore filter was efficient and allowed full recovery of the stoma, but was relatively slow (about the same duration as washing in the centrifuge). Separation on a column of beaded agarose gel (Sepharose CL-6B) was rapid and produced a homogenous suspension, but complete separation was not achieved in one passage down the column. The latter method is recommended as an alternative to centrifugation.

5. REFERENCES

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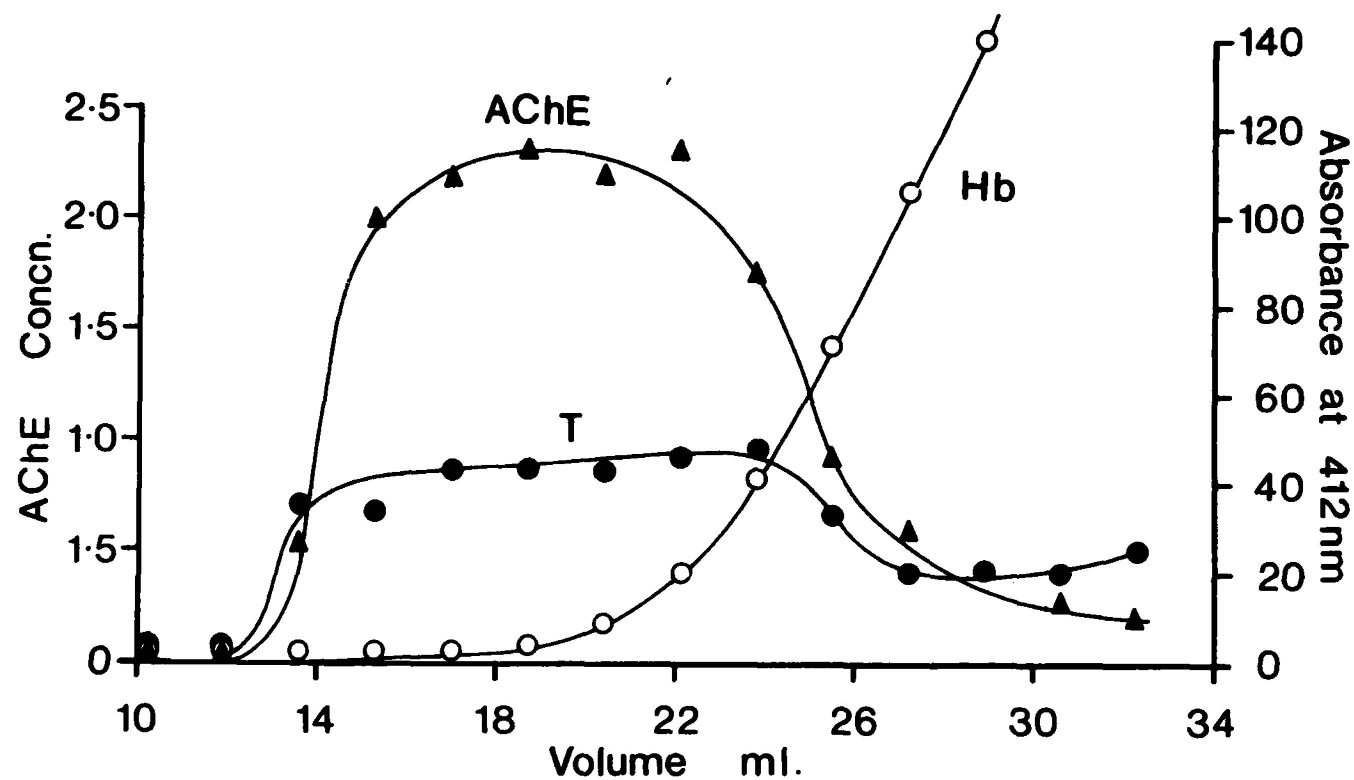

FIG. 1 - The separation of erythrocyte membrane particles from haemoglobin on a column of Sepharose CL-6B. Acetylcholinesterase (AChE) activity is shown on an arbitrary scale of activity on the left, Haemoglobin (Hb) concentration is expressed as the absorbance at 412 nm in a 10 mm cuvette and turbidity (T) is shown as apparent absorbance at 700 nm. For the latter parameter, the right hand scale must be reduced by a factor of 1/200.

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